

COMMONWEALTH OF AUSTRALIA

IN THE MATTER OF: Australian Patent
Application 696764 (73941/94). In the name of:
Human Genome Sciences Inc.

-and-

IN THE MATTER OF: Opposition thereto by
Ludwig Institute for Cancer Research, under
Section 59 of the Patents Act.

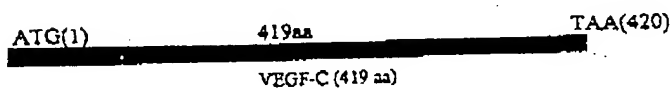
STATUTORY DECLARATION

I, Susan Power of Cell & Molecular Technologies, Inc., Phillipsburg, New Jersey, United
States of America, declare as follows:

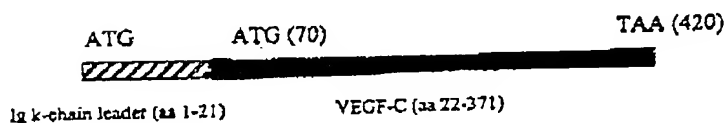
1. I am currently a Senior Molecular Biologist for Cell & Molecular Technologies, Phillipsburg, New Jersey. I have held this position since July, 1999. Prior to that appointment I was a post-doctorate research scientist, studying the molecular biology of retinoids and their role in limb bud development, at the University of Wisconsin, Madison, Wisconsin for two years. Prior to that appointment, I was a post-doctorate research scientist, studying the molecular biology of the transcription factor vHNF1 at the Pasteur Institute in Paris, France for four years. I received my Ph.D. in 1991 in Microbiology, from the National University of Ireland, Galway, Ireland.
2. The Patent Attorneys for Human Genome Sciences (HGS) requested that I perform the following experiments in order to determine whether the 350 amino acid form of VEGF-2 (corresponding to residues 70 to 419 of the 419 amino acid form of VEGF-2) fused in frame with a heterologous signal sequence would result in the expression and secretion of the protein from eukaryotic cells. They have also requested that all experiments that I conducted employ techniques routinely available by March, 1994. I have done this and the experiments I have conducted are described herein. Unless I state otherwise, all methods used herein were available prior to March, 1994.

3. To determine whether the 350 amino acid form of VEGF-2 could be secreted from cells when attached to a heterologous signal sequence, I transfected eukaryotic cells with expression vectors encoding the 419 amino acid form of VEGF-2, or the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence. I grew the transfected cells under conditions to allow the cells to express the gene products encoded by the vectors. At various time points, I collected both cell lysates and culture medium and assayed for the presence of VEGF-2, in order to determine if the cells were secreting VEGF-2. The presence of VEGF-2 in either the cell lysates or culture medium was determined by Western Blot analysis of the samples using a polyclonal antibody to VEGF-2, which recognizes both the precursor form and the processed form of VEGF-2

4. The design of the expression vectors used in the study is as follows:
419 amino acid form of VEGF-2 (followed by a STOP codon at position 420):



350 amino acid form of VEGF-2 linked to a heterologous signal sequence (followed by a STOP codon at position 420):



5. The nucleotide sequences encoding the 419 and the 350 amino acid forms of VEGF-2 were obtained directly from the American Type Culture Collection (ATCC). ATCC Deposit No. 97149 contains the nucleotide sequence encoding the 419 amino acid form of VEGF-2. ATCC Deposit No. 75698 contains the nucleotide sequence encoding the 350 amino acid form of VEGF-2. The nucleotide sequence encoding the 419 amino acid form of VEGF-2

was engineered to be flanked by an Eco RI site at the 5' end and a Not I site at its 3' end. The second construct contained the nucleotide sequence encoding the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, Ig k-chain leader signal sequence, a recognized signal sequence which was available as of March, 1994, and was engineered to contain a Bam HI site at its 5' end and a Not I site at its 3' end. The sequence of each of these constructs was confirmed to be correct and is detailed in Appendix I, attached hereto.

6. Each VEGF-2 construct was subcloned into the expression vector pCMV-I (see attached plasmid map, attached hereto as Appendix II), so that the VEGF-2 sequences were under the control of a CMV-I promoter, a promoter routinely used as of March, 1994. The 419 amino acid form of VEGF-2 was subcloned into the Eco RI/Not I sites of pCMV-I, while the 350 amino acid form of VEGF-2 fused in frame with a heterologous signal sequence, was subcloned into the Bam HI/Not I sites of pCMV-I (see plasmid map, Appendix II).
7. The two VEGF-2 constructs were transiently transfected in duplicate, using the lipofectin method, comparable methods were routinely used as of March, 1994, into the Human Embryonic Kidney cell line, HEK-293 tsA-0, a cell line which was also routinely used as of March, 1994. As a control for transfection efficiency, each construct was co-transfected with the vector pCMV- β -gal. The efficiency of transfection was determined by β -gal staining 48 hours after transfection. As a negative control the vector pCMV-I without an insert was transfected in parallel.
8. The transfection design is as follows:
 - 10 dishes transfected with: pCMV-I-VEGF-419;
 - 10 dishes transfected with: pCMV-I-signal sequence-VEGF-350;
 - 10 dishes transfected: pCMV-I;
 - 2 dishes transfected with: pCMV-I-VEGF-419 + pCMV- β -gal; and
 - 2 dishes transfected with: pCMV-I-signal sequence-VEGF-350 + pCMV- β -gal.

9. After transfection, DMEM medium containing 3% serum was added to the cells. Aliquots of cell extracts and conditioned medium were prepared from each transfection at: T₀ hours, T₁₆ hours, T₂₄ hours, T₄₈ hours and T₇₂ hours, in duplicate.
10. At the time of harvesting the cells and medium were treated as follows:
Medium: Harvested medium was concentrated 3 fold using Centricon 10 concentrator devices. One volume of 2 x PAGE loading dye was added to each sample.
Cell Extracts: The cells were harvested by trypsinization and collected by centrifugation. The cell pellet was resuspended and lysed in 250 μ l of 1x PAGE loading buffer.
11. To determine the transfection efficiency, dishes transfected with the pCMV- β -gal construct were fixed and stained for β -gal activity 48 hours after transfection. All dishes were found to have the same percentage of transfected cells (70%).
12. Each protein sample was subjected to Western analysis as outlined below. Prior to loading on to a 12% (w/v) Tris-Glycine SDS-Polyacrylamide gel, the samples were boiled for 5 minutes and cooled on ice. The two end lanes of each gel contained the appropriately sized molecular weight markers to estimate the migration rate of proteins predicted to run in the 16 to 85 kDa size range. The samples were electrophoresed according to standard conditions.
13. Following electrophoresis, the samples were transferred to a PVDF membrane. Each membrane was blocked by a one hour incubation in phosphate buffered saline (PBS) containing 3% Bovine Serum Albumin (BSA). The blot was then incubated at 4°C in PBS containing 0.1% BSA and 500ng/ μ l of purified rabbit anti-VEGF-2 antibody, a polyclonal antibody which recognizes the precursor form and the processed form of VEGF-2. After three 5 minute washes in PBS containing 0.1% Tween, the blot was incubated for 1 hour in PBS containing 0.1% BSA and a 1:3000 dilution of Goat Anti-Rabbit IgG

Horse Radish Peroxidase (HRP) conjugated antibody. The blot was washed three times for 5 minutes in PBS containing 0.1% BSA. The blot was developed with 2ml/blot of ECL detection reagent (obtained from Amersham) for one minute and then exposed directly to Polaroid films for approximately 5 seconds.

14. The result of the experiment is shown in Figure 1, attached hereto. The samples included in the figure are as follows:

Immunoblot analysis of VEGF derivatives transiently expressed in HEK293T cells

Lane	Pellet/Supe rn.	350aa -signal / 419aa	T (h) post-transfection
Gel 1			
1	P	419	24
2	S	419	24
3	P	350-signal	24
4	S	350-signal	24
5	P	350-signal	24
6	S	350-signal	24
7	P	negative control	24
8	S	positive control	48
Gel 2			
9	P	419	48
10	S	419	48
11	P	419	48
12	S	419	48
13	P	350-signal	48
14	S	350-signal	48

15	P	350-signal	48
16	S	350-signal	48
17	P	negative control	
18	S	negative control	
Gel 3			
19	P	419	72
20	S	419	72
21	P	419	72
22	S	419	72
23	P	350-signal	72
24	S	350-signal	72
25	S	350-signal	72
26	P	350-signal	72

15. The Western Blot analysis indicates that a doublet of approximately 30kDa was present in the medium collected from the transfection of both the 419 amino acid form of VEGF-2 and the 350 amino acid-VEGF-2 signal sequence constructs (see Figure 1). The secreted protein was visible beginning at 16 hours after transfection. The secreted product from cells containing the 419 amino acid construct and the 350 amino acid-VEGF2 signal sequence construct are approximately the same size.

AND I declare that all the statements made in this Declaration are of my own are true in every particular, and that all statements made on information and belief are believed to be true.

Sworn by the said Susan Power, Susan Power at
Phillipsburg, New Jersey, on this 3rd day of December 2000;
before me Maryann White
Notary Public

MARYANN WHITE
NOTARY PUBLIC, State of New York
No. 4883761
Qualified in Nassau County
Certification Filed in New York County
Commission Expires January 26, 2001

** TOTAL PAGE.07 **

VEGF-2 350aa +Signal Sequence:

[illegible]

VEGF-2 419aa Sequence:

EcoRI

+1 Met His Leu Leu Gly Phe Phe Ser Val Ala

1 GAATTCGTGG GTCCTTCCAC CATGCACTTG CTGGGCTTCT TCTCTGTGGC
CTTAAGCACC CAGGAAGGTG GTACGTGAAC GACCCGAAGA AGAGACACCG

SmaI
XmaI
AvaI
NruI

+1 Ala Cys Ser Leu Leu Ala Ala Ala Leu Leu Pro Gly Pro Arg Glu Ala Pro Ala

51 GTGTTCTCTG CTCGCCGCTG CGCTGCTCCC GGGTCCTCGC GAGGCGCCCG
CACAAGAGAC GAGCGGCGAC GCGACGAGGG CCCAGGAGCG CTCCGCGGGC

+1 Ala Ala Ala Ala Ala Phe Glu Ser Gly Leu Asp Leu Ser Asp Ala Glu Pro

101 CCGCCGCCGC CGCTTCGAG TCCGGA CTG ACCTCTCGGA CGCGGAGCCC
GGCGGCGGCG GCGGAAGCTC AGGCCTGAGC TGGAGAGCCT GCGCCTCGGG

DpnI
BglII

+1 Asp Ala Gly Glu Ala Thr Ala Tyr Ala Ser Lys Asp Leu Glu Glu Gln Leu

151 GACGCGGGCG AGGCCACGGC TTATGCAAGC AAAGATCTGG AGGAGCAGTT
CTGCGCCCGC TCCGGTGCCG AATACGTTCG TTTCTAGACC TCCTCGTCAA

SspI

+1 Leu Arg Ser Val Ser Ser Val Asp Glu Leu Met Thr Val Leu Tyr Pro Glu Tyr

201 ACGGTCTGTG TCCAGTGTAG ATGAATCAT GACTGTACTC TACCCAGAAT
TGCCAGACAC AGGTCACATC TACTTGAGTA CTGACATGAG ATGGGTCTTA

SspI DdeI

+1 Tyr Trp Lys Met Tyr Lys Cys Gln Leu Arg Lys Gly Gly Trp Gln His Asn

251 ATTGGA AAAT GTACAAGTGT CAGCTAAGGA AAGGAGGCTG GCAACATAAC
TAACCTTTTA CATGTTTACA GTCGATTCTT TTCCTCCGAC CGTTGTATTG

PstI

+1 Arg Glu Gln Ala Asn Leu Asn Ser Arg Thr Glu Glu Thr Ile Lys Phe Ala

301 AGAGAACAGG CCAACCTCAA CTCAAGGACA GAAGAGACTA TAAAATTTGC
TCTCTTGTC GGTGGAGTT GAGTTCTCTG CTTCTCTGAT ATTTTAAACG

DpnI
BglII

+1 Ala Ala Ala His Tyr Asn Thr Glu Ile Leu Lys Ser Ile Asp Asn Glu Trp Arg

351 TGCAGCACAT TATAATACAG AGATCTTGAA AAGTATTGAT AATGAGTGGG
ACGTCGTGTA ATATTATGTC TCTAGAACTT TTCATAACTA TTACTCACCT

SphI

+1 Arg Lys Thr Gln Cys Met Pro Arg Glu Val Cys Ile Asp Val Gly Lys Glu

401 GAAAGACTCA ATGCATGCCA CGGGAGGTGT GTATAGATGT GGGGAAGGAG
CTTTCTGAGT TACGTACGGT GCCCTCCACA CATATCTACA CCCCTTCCTC

NruI DraI AccI

+1 Phe Gly Val Ala Thr Asn Thr Phe Phe Lys Pro Pro Cys Val Ser Val Tyr

451 TTTGGAGTCG CGACAAACAC CTTCTTTAAA CCTCCATGTG TGTCCGTCTA
AAACCTCAGC GCTGTTTGTG GAAGAAATTT GGAGGTACAC ACAGGCAGAT

AccI PstI

+1 Tyr Arg Cys Gly Gly Cys Cys Asn Ser Glu Gly Leu Gln Cys Met Asn Thr Ser

501 CAGATGTGGG GGTGCTGCA ATAGTGAGGG GCTGCAGTGC ATGAACACCA
GTCTACACCC CCAACGACGT TATCACTCCC CGACGTCACG TACTTGTTGGT

	+1	-Ser Thr Ser Tyr	Ddel Leu Ser Lys	Thr Leu Phe Glu Ile Thr Val Pro Leu Ser	
551		GCACGAGCTA CCTCAGCAAG ACGTTATTTG AAATTACAGT GCCTCTCTCT		CGTGCTCGAT GGAGTCGTTC TGCAATAAAC TTAAATGTCA CGGAGAGAGA	
	+1	Gln Gly Pro Lys Pro Val Thr Ile Ser Phe Ala Asn His Thr Ser Cys Arg			
601		CAAGGCCCCCA AACCAAGTAAC AATCAGTTTT GCCAATCACCA CTTCCTGCCG		GTTCCGGGGT TTGGTCATTG TTAGTCAAAA CGGTAGTAGT GAAGGACGGC	
	+1	Arg Cys Met Ser Lys Leu Asp Val Tyr Arg Gln Val His Ser Ile Ile Arg Arg			
651		ATGCATGTCT AAACCTGGATG TTTACAGACA AGTTCATTCC ATTATTAGAC		TACGTACAGA TTTGACCTAC AAATGTCTGT TCAAGTAAGG TAATAATCTG	
	+1	Arg Ser Leu Pro Ala Thr Leu Pro Gln Cys Gln Ala Ala Asn Lys Thr Cys			
701		GTTCCCTGCC AGCAACTACTA CCACAGTGTC AGGCAGCGAA CAAGACCTGC		CAAGGGACGG TCGTTGTGAT GGTGTCACAG TCCGTCGCTT GTTCTGGACG	
	+1	Pro Thr Asn Tyr Met Trp Asn Asn His Ile	PstI Cys Arg Cys	Leu Ala Gln Glu	
751		CCCACCAATT ACATGTGGAA TAATCACATC TGCAGATGCC TGGCTCAGGA		GGGTGGTTAA TGTACACCTT ATTAGTGTAG ACGTCTACGG ACCGAGTCCT	
	+1	Glu Asp Phe Met Phe Ser Ser Asp Ala Gly Asp Asp Ser Thr Asp Gly Phe His			
801		AGATTTTATG TTTTCTCTCG ATGCTGGAGA TGAICTAACA GATGGATTCC		TCTAAAAATAC AAAAGGAGCC TACGACCTCT ACTGAGTTGT CTACCTAAGG	
	+1	His Asp Ile Cys Gly Pro Asn Lys Glu Leu Asp Glu Glu Thr Cys Gln Cys			
851		ATGACATCTG TGGACCAAAC AAGGAGCTGG ATGAAGAGAC CTGTCACTGT		TACTGTAGAC ACCTGGTTTG TTCCTCGACC TACTTCTCTG GACAGTCACA	
	+1	Val Cys Arg Ala Gly Leu Arg Pro Ala Ser Cys Gly Pro His Lys Glu Leu	PvuII PstI		
901		GTCTGCAGAG CGGGGCTTCG GCCTGCCAGC TGTGGACCCC ACAAGAAGCT		CAGACGTCTC GCCCCGAAGC CGGACGGTCG ACACCTGGGG TGTTTCTGA	
	+1	Leu Asp Arg Asn Ser Cys Gln Cys Val Cys Lys Asn Lys Leu Phe Pro Ser Gln			
951		AGACAGAAAC TCATGCCAGT GTGTCTGTAA AAACAAACTC TTCCCAGCC		TCTGTCTTTG AGTACGGTCA CACAGACATT TTTGTTTGAG AAGGGGTCGG	
	+1	Glu Cys Gly Ala Asn Arg Glu Phe Asp Glu Asn Thr Cys Gln Cys Val Cys			
1001		AATGTGGGGC CAACCGAGAA TTTGATGAAA ACACATGCCA GTGTGTATGT		TTACACCCCG GTTGGCTCTT AAACACTTTT TGTGTACGGT CACACATACA	
	+1	Lys Arg Thr Cys Pro Arg Asn Gln Pro Leu Asn Pro Gly Lys Cys Ala Cys			
1051		AAAAGAACCT GCCCCAGAAA TCAACCCCTA AATCCTGGAA AATGTGCCTG		TTTTCTTGGA CGGGGTCTTT AGTTGGGGAT TAGGACCTT TTACACGGAC	
	+1	Cys Glu Cys Thr Glu Ser Pro Gln Lys Cys Leu Leu Lys Gly Lys Lys Phe His			
1101		TGAATGTACA GAAAGTCCAC AGAAATGCTT GTTAAAAGGA AAGAAGTTCC		ACTTACATGT CTTTCAGGTG TCTTTACGAA CAATTTTCCT TTCTTCAAGG	
	+1	His His Gln Thr Cys Ser Cys Tyr Arg Arg Pro Cys Thr Asn Arg Gln Lys	PvuII		
1151		ACCACCAAAC ATGCAGCTGT TACAGACGGC CATGTACGAA CCGCCAGAAG		TGGTGGTTTTG TACGTCGACA ATGTCTGCCG GTACATGCTT GGCGGTCTTC	
	+1	Ala Cys Glu Pro Gly Phe Ser Tyr Ser Glu Glu Val Cys Arg Cys Val Pro			
1201		GCTTGTGAGC CAGGATTTTT ATATAGTGAA GAAGTGTGTC GTTGTGTCCC		CGAACACTCG GTCCTAAAAG TATATCACTT CTTACACAG CAACACAGGG	

Ddel
~~~~~  
EagI  
~~~~~  
NotI
~~~~~

+1 Pro Ser Tyr Trp Lys Arg Pro Gln Met Ser ...  
1251 TTCATATTGG AAAAGACCAC AAATGAGCTA AGCGGCCGCG  
AAGTATAACC TTTTCTGGTG TTTACTCGAT TCGCCGGCGC

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# STRAIN SHEET

STRAIN CMV I

STORAGE NUMBER 11:64

## PEDIGREE:

-CMV I was constructed in the pSV7 (nee pSG5 from Stratagene, with an expanded polylinker) backbone by replacing the SV40 promoter from pSV7 with the CMV promoter/enhancer/intron, via 5' Sal I/Xho I (sites destroyed) and 3' Hind III.

-CMV I is 4613 bp.

-CMV I uses ampicillin resistance.

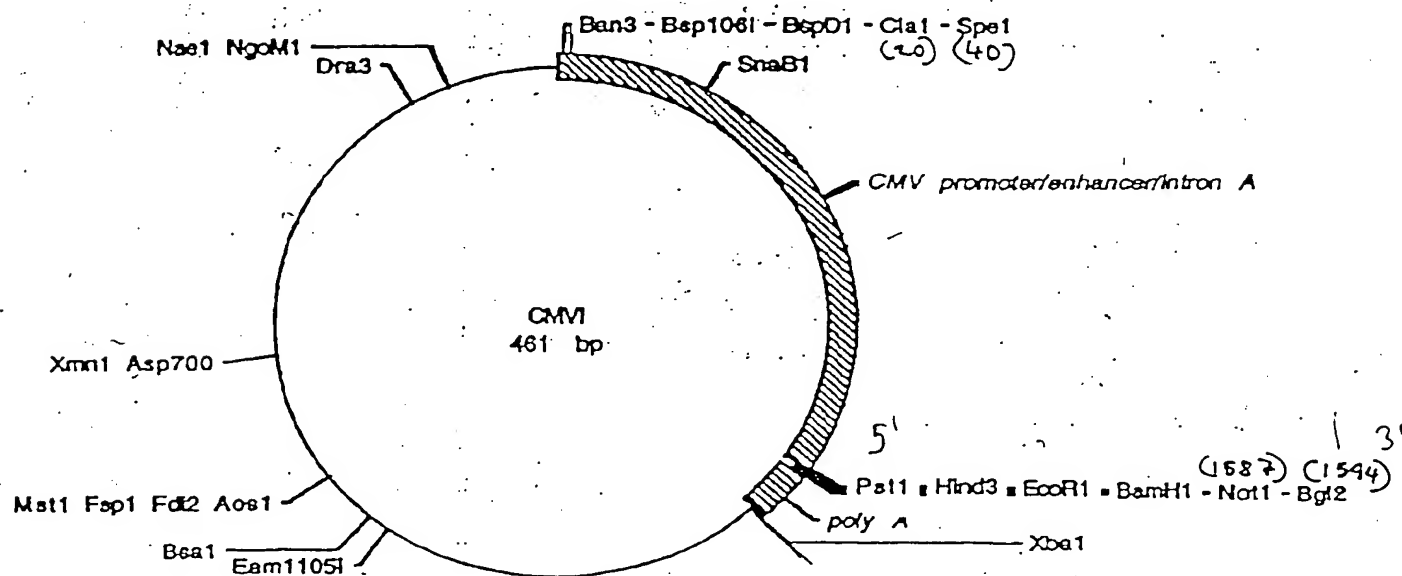
-CMV promoter/enhancer + Intron A: nt #'s 1-1566.

-Polylinker: nt #'s 1566-1597 (5' - Hind III - Eco RI - Bam HI - Not I - Bgl II - 3').

-SV40 polyA addition sequence: nt #'s 1598-1745.

-If for some reason you want to remove the SV40 polyA addition sequence, you can cut with either Sal I or Xba I (these 2 sites border the 3' end of this sequence).

## GROWTH REQUIREMENTS:



Appendix II

kDa

-148

-98

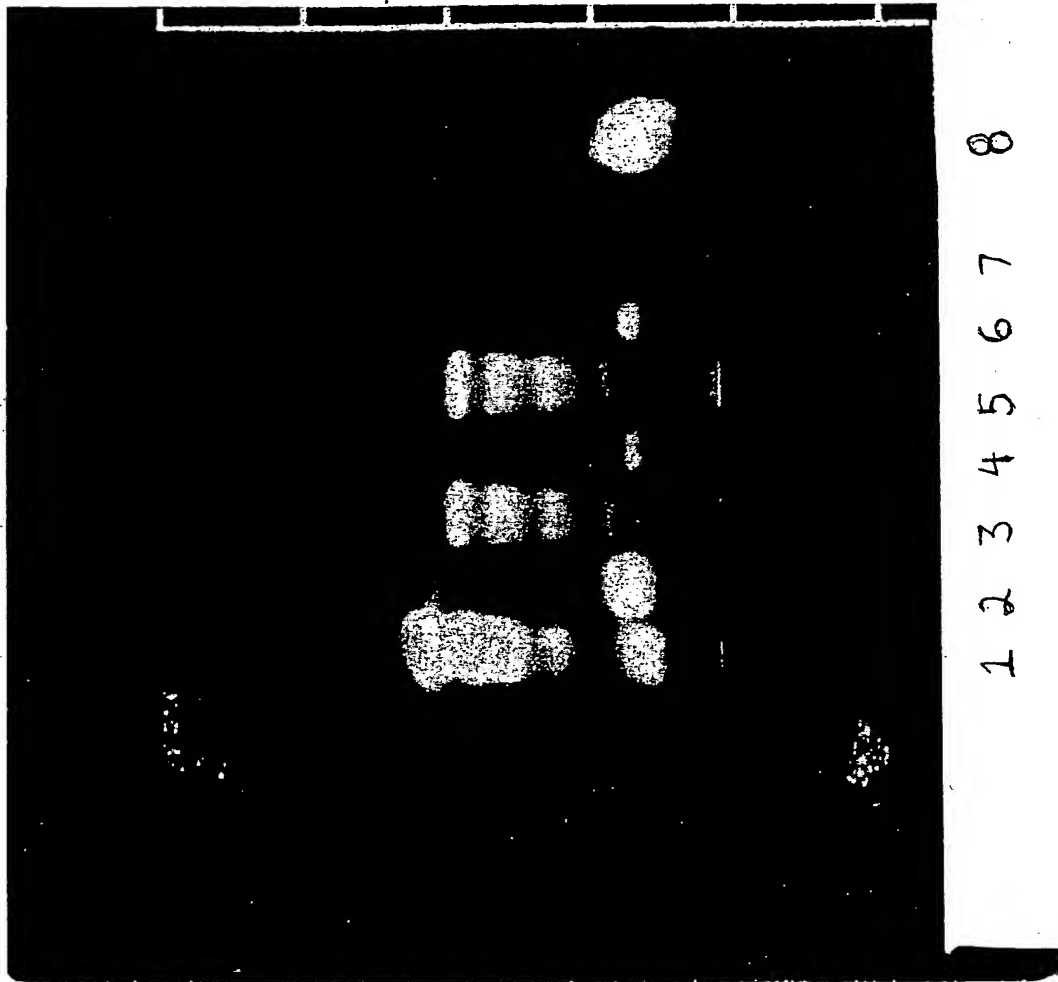
-64

-50

-36

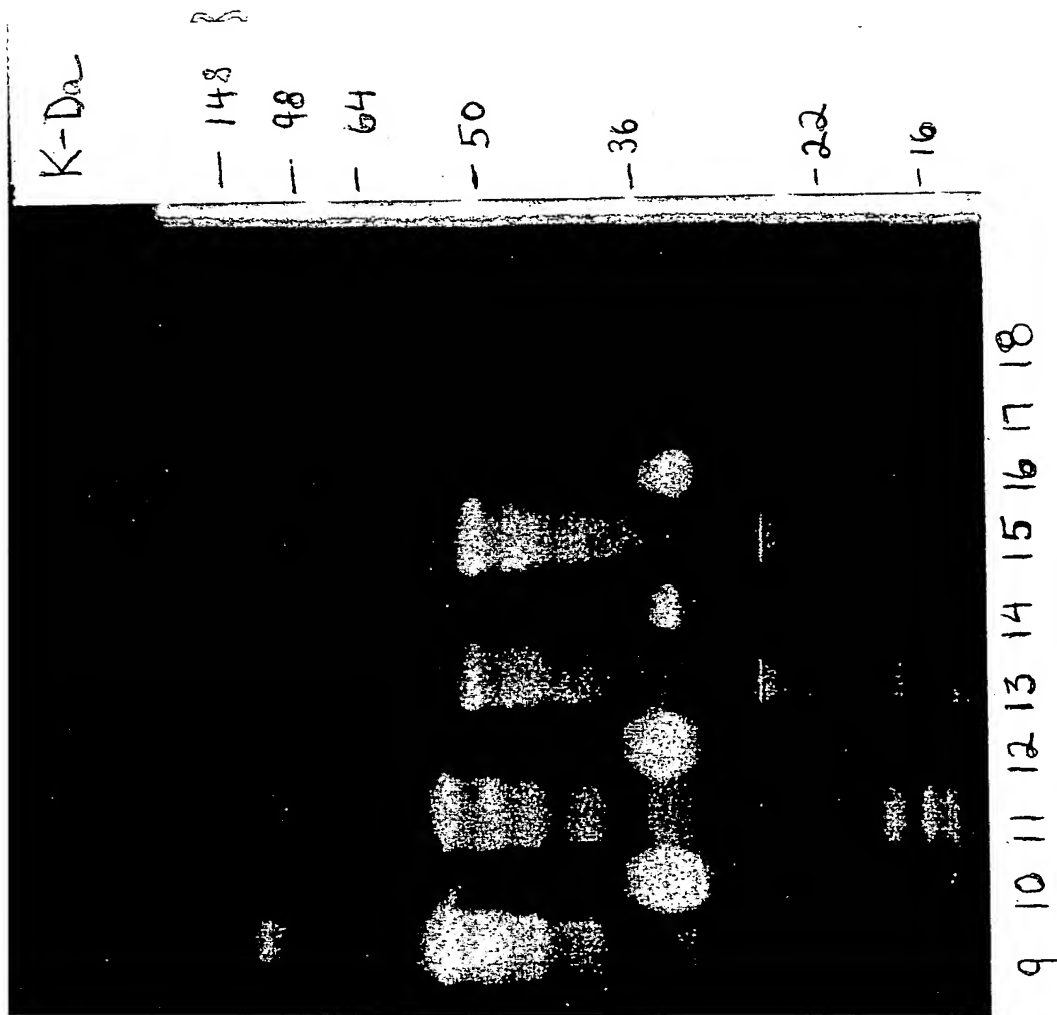
-22

-16

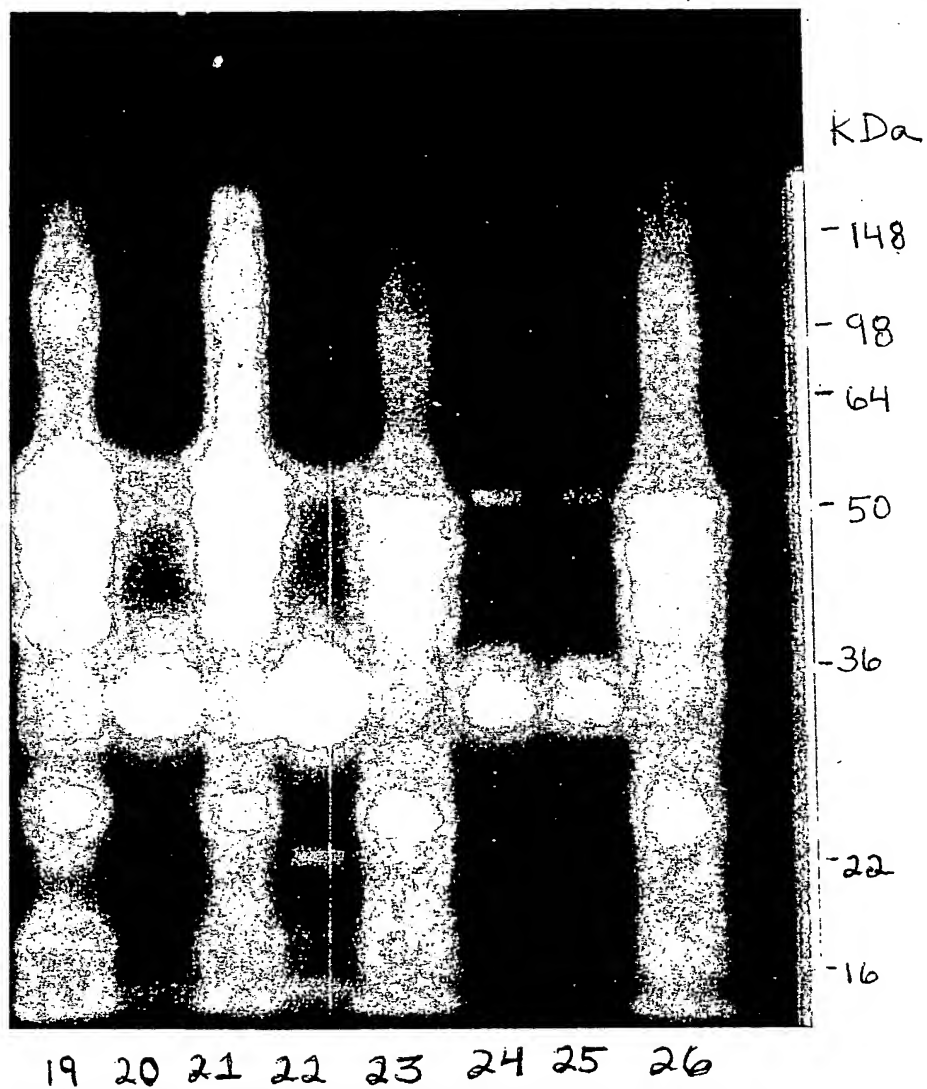


1 2 3 4 5 6 7 8

Power Declaration  
Figure 1 Gel 1



Power Declaration  
 Figure 1 - Gel 2



Power Declaration  
Figure 1 - Gel 3